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Solid-state NMR analysis of the orientation and dynamics of epigallocatechin gallate, a green tea polyphenol, incorporated into lipid bilayers

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Catechins are the principle polyphenolic compounds in green tea; the four major compounds identified are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg) and epigallocatechin gallate (EGCg). Tea catechins tend to attach externally to their targets, such as viral envelopes, cell membranes, or the surface of low-density lipoproteins. In order to further our understanding of the molecular mobility of these compounds in cells, we examined the interaction of tea catechins with lipid membranes using solid-state NMR techniques. Our previous work indicated that the EGCg molecule is incorporated into lipid bilayers in a unique orientation. However, the detailed configuration, orientation, and dynamics of EGCg in lipid bilayers have not been well-characterized. Here, we investigated the orientation and dynamics of EGCg incorporated into multi-lamellar vesicles (MLVs) and bicelles using solid-state NMR spectroscopy.

Keywords: tea catechins; epigallocatechin gallate; lipid membrane; solid-state NMR; liposome; bicelle

Introduction

Tea catechins function biologically as antioxidants,[1,2] antitumor agents,[3–6] and antibiotics,[7] and have been investigated by *in vitro* experiments using cultured cells or bacteria. The degree of activity observed is dependent on the *in vitro* system. These differences in biological activities among the tea catechins may partly be attributed to the amount of compound incorporated into the lipid bilayers. We found that the affinity of tea catechins to lipid bilayers is characterized by (i) the number of hydroxyl groups on the B-ring, (ii) the presence of the galloyl moiety, and (iii) the stereochemical structure of each catechin.[8] The salt concentration in an aqueous medium, the electric charges on the membrane, and the presence of other catechins, are also important factors affecting the affinity of tea catechins for lipid bilayers.[9] These experimental findings suggest that tea catechins interact with the phospholipid domains of membranes. To help elucidate their molecular mobility in the cell, we examined the interaction of tea catechins with lipid membranes using solid-state NMR techniques. Our previous work indicated that the epigallocatechin gallate (EGCg) molecule is incorporated into lipid bilayers by adopting an unique orientation, perturbing head-group motion and the conformation of the phospholipids.[10] However, the orientation and motion of EGCg in lipid bilayers have not been well-characterized. In this study, we investigated the orientation and dynamics of EGCg incorporated into multi-lamellar vesicles (MLVs) and bicelles using solid-state ^{31}P and ^{2}H NMR spectroscopy.

Experimental

Sample preparation

[4-^{2}H]EGCg (Fig. 1) deuterated at the fourth position in the C-ring was synthesized from EGCg (Mitsui Norin, Shizuoka, Japan), as reported previously.[11] To incorporate EGCg into liposomes, [4-^{2}H]EGCg, and dimyristoylphosphatidylcholine (DMPC) (Sigma, St Louis, MO) were dissolved in chloroform–methanol (1 : 1, v/v) with a [4-^{2}H]EGCg-to-DMPC molar ratio of 1 : 20. The solvent was subsequently evaporated *in vacuo*, followed by hydration with deuterium–depleted water (Wako Pure Chemical Industries, Osaka, Japan). The sample, in a glass sample tube, was subjected to repeated freeze–thaw cycles under a stream of N_2 gas. Bicelles were made of DMPC and dihexanoylphosphatidylcholine (DHPC) (Sigma) with a long chain to short chain lipid molar ratio of 3 : 1. First, [4-^{2}H]EGCg and DHPC were mixed at a [4-^{2}H]EGCg -to-DHPC molar ratio of 1 : 5. The solvent was subsequently evaporated *in vacuo*, followed by hydration with deuterium-depleted water (hydration ratio: 25 w/v% of DHPC). Next, DMPC and deuterium-depleted water (hydration ratio: 25 w/v% of phospholipids) were added to this mixture. The glass sample tube was subjected to a heating (60 °C)–cooling (0 °C) cycle, repeated 20 times under a stream of N_2 gas.

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Solid-state NMR sprectroscopy

All solid-state NMR experiments were carried out using a CMX-400 Infinity NMR spectrometer (Varian Associates Inc. Fort Collins, CO). ^{31}P NMR spectra were recorded at a resonance frequency of 161.15 MHz. A 90° excitation pulse and a recycle delay of 4.3 μs and 2 s, respectively, were used. Spectra were measured at 20, 25, 30, and 40 °C; typically, 200 scans were collected. The chemical shift value of 85% H_3PO_4 was used as a reference for the ^{31}P NMR spectra at 0 ppm. ^{2}H NMR spectra were recorded at a resonance frequency of 61.4 MHz with the quadrupole echo pulse sequence ($90° - \tau - 90° - \tau_1 -$ echo; $\tau \neq \tau_1$). A 90° pulse length of 3 μs was used. Spectra measured at 10, 20, 30, and 40 °C (MLVs), or 40 °C (bicelles). Pulse intervals (τ) of 30, 60, and 90 μs (MLVs), or 60, 90, and 120 μs (bicelles), were used for measuring the motion of [4-^{2}H]EGCg incorporated into lipid bilayers. The number of scans was typically 180 000 (MLVs) or 60 000 (bicelles). The chemical shift value of D_2O with $CuCl_2$ was used as a reference for the ^{2}H NMR spectra (0 ppm).

Results and Discussion

Solid-state NMR has been used to elucidate the orientation and motion of molecules.[12] In particular, ^{2}H NMR spectra provide direct information on the orientation and dynamics of site-specifically labeled C–^{2}H bonds,[13,14] as in the case of [4-^{2}H]EGCg (Fig. 1). Figure 2 shows the temperature dependence of ^{2}H NMR spectra of [4-^{2}H]EGCg incorporated into pure DMPC lipid bilayers of MLVs near the gel to liquid-crystalline phase transition temperature (T_c, 23 °C). These spectra show the mixture of narrow isotropic and broad powder patterns in the temperature range 10–40 °C Fig. 2(a–d)). In particular, the broad component of ^{2}H NMR spectra become narrower at temperatures above 30 °C (Fig. 2(c)) because molecular mobility increases above T_c (Fig. 2(c) and (d)) and decreases below T_c (Fig. 2(a) and (b)). These results indicate that the EGCg molecules which show broad ^{2}H NMR pattern are incorporated into the DMPC membrane in this temperature range. On the other hand, the isotropic signal can be attributed to free EGCg rather than to signals from HO^{2}H since deuterium-depleted water was used in these experiments. Furthermore, small changes in the powder patterns of [4-^{2}H]EGCg were observed by varying the pulse intervals (τ) from 30 (Fig. 2(e)) to 60 (Fig. 2(f)) to 90 μs (Fig. 2(g)). As the τ value increased, the line width decreased, suggesting that heterogeneous molecular states with different motions coexist. Broad components in Fig. 2(e), (f), and (g) become axially symmetric at temperatures above 30 °C, indicating that the EGCg molecules rotate about a unique axis as a main dynamics. Further, the line widths slightly decreased as the pulse intervals increase, indicating that highly mobile components of this sample with long-relaxation times (T_2) became significant at a longer-pulse interval. The small heterogeneity of catechin molecules suggests that various types of EGCg interactions with lipid bilayers exist in the membrane-bound state. Nevertheless, it is noted that the bound structures of [4-^{2}H]EGCg in the lipid membrane are nearly identical but have axially symmetric motions with slightly different wobbling amplitudes.

Bicelles consist of short- and long-chain phospholipids, and the surfaces of bicelles spontaneously align parallel to the magnetic field above T_c.[15,16] Bicelle surfaces are magnetically oriented over a wide range of concentrations, compositions, buffer pHs, and temperatures because of the diamagnetic nature of bicelles.[17] Since bicelle surfaces orient parallel to the magnetic field, a bound

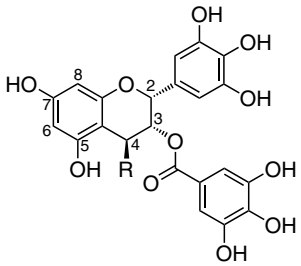

Figure 1. Structures of EGCg (R = H) and [4-^{2}H]EGCg (R = ^{2}H).

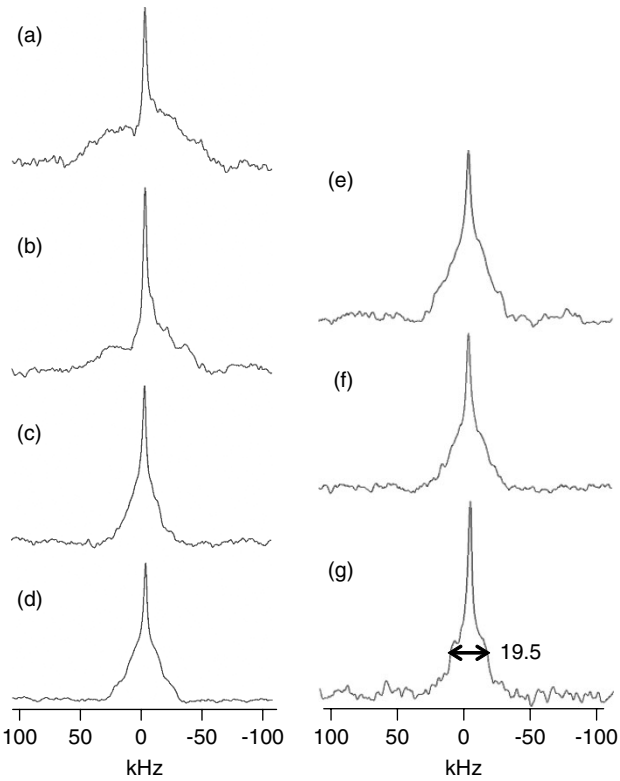

Figure 2. Temperature dependence of the powder patterns of solid-state ^{2}H NMR spectra for [4-^{2}H]EGCg in MLVs (τ = 30 μs): (a) 10 °C; (b) 20 °C; (c) 30 °C; and (d) 40 °C. Powder patterns obtained by varying pulse intervals at 40 °C; (e) τ = 30 μs; (f) τ = 60 μs; and (g) τ = 90 μs. The MLVs consist of DMPC (T_c = 23 °C). The chemical shifts of D_2O with $CuCl_2$ served as a reference for the ^{2}H NMR spectra. The splitting of the perpendicular component is approximately 19.5 kHz above T_c.

molecule would also be oriented with respect to the magnetic field.[18–22] Using such a bicelle system, it is possible to obtain information with high resolution and sensitivity on the orientation of molecules incorporated into membranes. At T_c, the interactions of short-chain phosphatidylcholine (dihexanoyl or diheptanoyl) with long-chain (dimyristoyl, dipalmitoyl, or distearoyl) phosphatidylcholine would predominantly have characteristics of the long-chained component.[23,24] Thus, mixtures of DHPC and DMPC in water would be expected to form discoidal aggregates in which a flat DMPC bilayer is stabilized by the DHPC rim.[25]

Using solid-state ^{31}P NMR spectrometry, we confirmed that the surfaces of bicelles orient parallel to the magnetic field (data not shown). Below T_c, an isotropic peak was observed at approximately 0 ppm. Two peaks gradually grew above T_c, showing the alignment

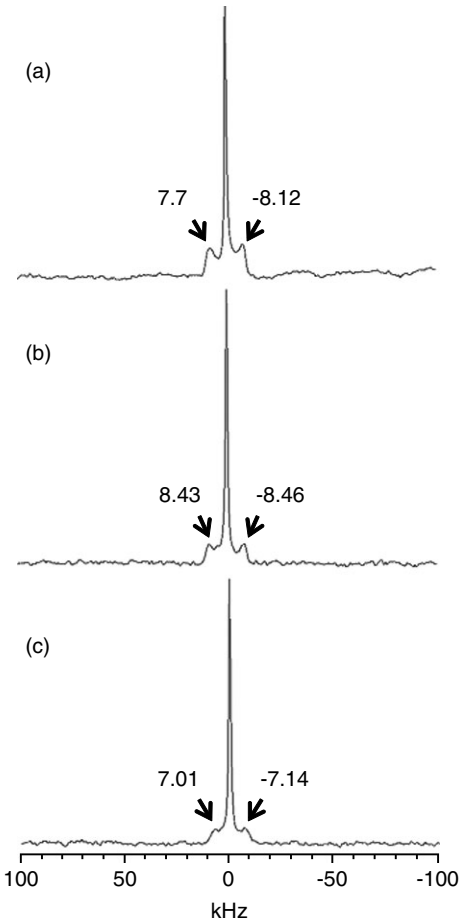

Figure 3. Solid-state ^{2}H NMR spectra in the oriented bicelles using various pulse intervals at 40 °C: (a) $\tau = 60$ μs; (b) $\tau = 90$ μs; and (c) $\tau = 120$ μs. The splitting of the quadrupole doublet is approximately 15.5 kHz.

of the bicelles along the magnetic field. Peaks corresponding to DHPC and DMPC were observed at −4.0 ppm and −8.5 ppm, respectively. Further, the heights of the two peaks (DMPC and DHPC) correspond to the molar ratio 3 : 1. Thus, the membrane surfaces of these bicelles were parallel to the magnetic field.

Solid-state ^{2}H NMR measurements were carried out to examine the orientation of [4-^{2}H]EGCg with respect to the lipid membrane. ^{2}H spectra of bicelles show two types of peaks: an intense central signal and a quadrupole doublet (Fig. 3). Peaks in the center region could be due to residual HO^{2}H or free [4-^{2}H]EGCg molecules in aqueous solution.[26] However it is improbable that this strong signal is due to a ^{2}H NMR peak from water because deuterium-depleted water was used in these experiments. In addition, we previously showed that not all EGCg added is incorporated into liposomes.[8] Therefore, the central signal is probably due to free, nonmembrane-bound [4-^{2}H]EGCg similar as in the case of liposome system. It is noted that that the fraction of unbound [4-^{2}H]EGCg is higher than the fraction, bound to liposomes. The doublet component corresponding to oriented membrane-bound EGCg shows splitting of *ca* 15.5 kHz. This splitting is nearly the same as the width of the perpendicular component (*ca* 19.5 kHz) in the MLV (Fig. 2(g)), strongly suggesting that the C–^{2}H bond rotates about the axis perpendicular to the magnetic field. Since the surface of the bicelle is parallel to the magnetic field, [4-^{2}H]EGCg molecules rotate about the bilayer normal with a constant tilt angle to the axis. Similar dynamic pictures have been observed for other membrane-bound molecules such as melittin (transmembrane type)[20] and dynorphin (surface type).[27] Furthermore, the intensity of the central lines increased, and the splitting of the orientated spectra decreased as the pulse interval increased. These findings suggest that the strong center peak is due to free EGCg and that the affinity of [4-^{2}H]EGCg for membranes is lower than the case of liposome-bound EGCg.

In the dynamic model described above, the C–^{2}H vector in [4-^{2}H]EGCg is rotating about the bilayer normal, and the ^{2}H quadrupole splitting (Dq) of the perpendicular component is given by Eqn (1).

$$Dq = D_Q S_{CD} \tag{1}$$

where

$$D_Q = \frac{3}{4}\frac{e^2qQ}{h} \text{ and } S_{CD} = \frac{1}{2}(3\cos^2\beta - 1).$$

D_Q is the ^{2}H quadrupole splitting of the perpendicular component in the static state, and e^2qQ/h is the quadrupole coupling constant which, for the sp^3-hybridized C–^{2}H pair, is 167 kHz. The experimentally-derived D_Q value for [4-^{2}H]EGCg is 125 kHz.[10] S_{CD} is the orientation order parameter and β is the tilt angle of the C–^{2}H vector to the rotary axis. The experimentally obtained D_Q value of [4-^{2}H]EGCg (Fig. 2(d)) incorporated into MLVs provides a value of 19.5 kHz. By substituting this value into Eqn (1), the tilt angle of the C–^{2}H vector to the rotary axis (β) is evaluated to be $\beta = 48^\circ$.

It is, therefore, probable that the hydrophobic domain of EGCg is liable to protrude into lipid bilayers and contact the inner lipophilic region.[8] Further, EGCg can diffuse laterally and rearrange its molecular orientation about the bilayer normal as an unique axis by keeping the tilt angle to the bilayer normal constant. Thus, EGCg molecules bound to lipid bilayers exhibit an above mentioned dynamic behavior. Consequently, molecules designed using the basic EGCg framework should be effective at their designed functions since they will be able to bind to or be incorporated into lipid membranes. Tachibana et al. have identified a receptor protein for EGCg.[28] EGCg must interact with the cell membrane before binding to the receptor. Since our results indicate that EGCg interacts with membrane surface and moves freely on the membrane surface, it would be able to bind to its target receptor.

The present study shows that the C–^{2}H axis of [4-^{2}H]EGCg is tilted 48° to the rotary axis of [4-^{2}H]EGCg. Magnetically oriented [4-^{2}H]EGCg-bicelle systems indicate that the rotating axis of [4-^{2}H]EGCg is perpendicular to the magnetic field. Further, the results show that catechin molecules incorporated into lipid bilayers have an axially symmetric motion with slightly different wobbling amplitudes. Polyphenols, including catechins, have hydroxyl groups in the flavane skeleton and are widespread in nature; for example, they are found in vegetables and fruits. The present study provides evidence of the interaction between tea catechins and membranes, and suggests that the intracellular dynamic state of catechins correlates to their biological activity.

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